Online portable microcantilever biosensors for Salmonella enterica serotype enteritidis detection

UNIVERSITÀ DEGLI STUDI DI TORINO

Online, Rapid and Portable Microcantilever Biosensors for Food Safety Monitoring

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Abstract. *The micro- and nano-technologies coupled with a deep knowledge of organic/inorganic interfaces guarantee an exceptional sensitivity and specificity of the sensor, while the lab-on-a-chip platform reduces assay times and limits sampling and/or sample preparation, providing compact and portable objects. Therefore, the development of innovative biosensors such as antibody-immobilized microcantilevers can overcome the evident limits of nowadays technologies, such as time consuming, expensiveness, difficult automation, low sensitivity, accuracy and precision for quantitative methods.*

The present study proposes two device designs for the detection of food pathogens, exploiting an antibody-immobilized microcantilever biosensors, a novel class of mass detectors. For the first one, we integrated the mechanical sensors on a microfluidic platform (lab-on-a-chip) to perform online analysis, directly in liquid environment. We showed that our portable biosensors could easily detect the presence of pathogenic bacteria such as Salmonella enterica serotype enteritidis in concentration 10^5 cfu/mL in just 40 minutes, without any enrichment and/or sample preparation. To increase the mass sensitivity of our analysis, we also fabricated microstructures optimized for vibrating in vacuum environment. Using a dip-and-dry technique, we showed that, in such configuration, the experimental limit of detection is as low as 10^3 cfu/mL. Due to the extremely small volumes needed, our biosensors operating in vacuum have the potentiality of detecting the presence or absence of a single cell.

Keywords. *Microcantilever, Biosensor, Pathogen Detection.*

Introduction

Traditional microbiological techniques are time consuming and in the case of pathogen detection they may require up to 7 days for the full identification of the species. These approaches are cost efficient, sensitive, and give both qualitative and quantitative information on the number and the nature of the microorganisms, but they are restricted by assay time, due to the initial enrichment needed in order to detect low concentrations of pathogens that typically occur in food and water. Moreover it is well accepted that cells that are stressed or injured often are not able to grow in the selective media used, thereby false negative results may occur. Most widely used biochemical methods for pathogen detection are polymerase chain reaction (PCR) and enzyme-linked immunosorbent assay (ELISA). Both approaches are well established, but frequently require extensive sample cleaning and purification; furthermore, being label-dependent, complex read-out instrumentations and specialized laboratories are needed (Ivnitski et al., 1999; Leonard et al., 2003). For all these reasons it is necessary to optimize and validate rapid methods that will enable a fast and reliable detection of foodborne pathogens, without the need for pre-concentration or constant attention of laboratory personal for preparation of samples and interpretation of results. These kinds of biosensors are intrinsically safe since they require very small quantity of pathogen and they are integrated in sealed automated microfluidic platform.

A number of label-free biosensors that are capable of monitoring in real-time both the presence of antigen and the quantification of the target in term of absolute mass and/or concentration have been proposed so far. At present, the most interesting technologies are: Surface Plasmon Resonance (SPR), Quartz Crystal Microbalance (QCM) and MicroCantilever (MC) (Skottrup et al., 2008).

Microcantilever (MC) based mass detectors probably represent the most promising class of label free biosensor platform for in situ measurements (Waggoner & Craighead, 2007). Ilic et al. (2001) first reported the detection of single *Escherichia coli* (EC) cells using a cantilever array vibrating in vacuum. Maraldo et al. (2007) extended the work of Ilic and co-workers, developing a composite, self-excited lead zirconate titanate (PZT)- glass millimeters size cantilever coated with anti-EC antibodies to detect the pathogen, EC O157:H7. They recently obtained impressive sensitivities for measurements both in buffered salt solutions (10 cfu/mL) and ground beef (100 cfu/mL). Few research groups reported on *Salmonella spp.* detection. Recently, Fu et al. (2007) reported the use of magnetostrictive microcantilever surface activated with phages to detect *Salmonella typhimurium* in liquid environment with a concentration of $5x10^8$ cfu/mL. The latter experimental set-up was applied by the same group to the detection of *Bacillus anthracis* spores in water, reaching a detection limit of 10^5 cfu/mL (Li et al., 2009). *S. typhimurium* cells were also successfully detected by Zhu et al. (2007) using a 3 mm long piezoelectric cantilever with a detection concentration sensitivity of $5x10^3$ cfu/mL. The successful detection of *Salmonella enterica* (Weeks et al., 2003) and EC O157:H7 (Zhang & Ji, 2004) was also reported using MC in the static mode, where the beam is deflected due to a change in the surface stress upon binding on cantilever surface.

In this study, we report on the successful use of microcantilever array for the detection of *Salmonella enterica* serotype *enteritidis* both in vacuum and liquid environment, with concentrations ranging from 10^8 colony forming units (cfu)/mL to 10^3 cfu/mL. Due to the small volumes used and the high sensitivity of the mechanical transduction, we were able to register a signal corresponding to just few immobilized cells. The use of the optimized biosensor allows a rapid detection of low numbers of this foodborne pathogen in a short time, allowing its use at

industrial level in order to guarantee food safety. Food industries will benefit of this tool that will allow a better control of foodstuffs and at the same time will shorten the time required to obtain the results of the analysis.

Materials and Methods

Concerning the device designed and used for "in vacuum" measurements, 4 × 4 mm^2 samples from silicon on insulator (SOI) wafers containing array of 6-10 microcantilevers (length 400-500 µm, width 50-60 µm and thickness 6-7 µm) were fabricated. As described in details by Canavese et al. (2007) the microstructures were made through an innovative combination of surface and bulk micromachining process. For in liquid environment measurements larger SOI chips (14 x 28 mm^2) containing two separate suspended microstructures were fabricated. The cantilevers for in liquid measurement (planar dimensions ranging from 1500x1000 μm^2 to 600x200 μm^2 and 6 µm thick) were first machined by the above cited fabrication process. Then, on the bottom side the device chips were anodically bonded to a pyrex cover, which contains the micro fluidic channels. On the top side the device was sealed with an home-made O-ring PDMS interconnection in order to make inlet and outlet reversible connection (Quaglio et al. 2008). See Fig. 1 for a picture of both devices.

Cantilever vibrational characteristics were measured with the *optical lever* technique: a piezoelectric actuator for the excitation and a position sensitive detector (PSD) as detector were used. For in vacuum measurements cantilevers were placed in a cell evacuated by a series of a membrane and turbomolecular pump (MINI-Task System, Varian Inc. Vacuum Technologies); for in liquid measurements a Syringe Pump by Harvard Apparatus was used in order to pump the liquid inside the platform by means of a tubing connection. The measurement procedure as well as the fitting of data with a Lorentzian curve were performed by a software in LABVIEW® environment.

Immobilization of bacteria was performed thanks to the anchoring of antibodies specific for all *Salmonella* spp. in an oriented manner on protein G activated cantilever surface. The Si MC were first thermally oxidized and secondly functionalized with 3-aminopropyl-triethoxysilan and glutaraldehyde solution. Then, functionalized MCs were washed and incubated in a protein G solution (50 µg/mL in PBS) for 2h at room temperature using an orbital shaker. Finally, 20 µL of Polyclonal antibodies HRP (Sacco, Milan, Italy), specific for all *Salmonella* spp., was dropped on MCs. The incubation of 2h was performed at room temperature in a chamber with controlled humidity, to avoid evaporation of solution. All chemicals and buffer components were purchased from Sigma-Aldrich. Recombinant Protein G, purified from Streptococcus, was from PIERCE.

Salmonella enterica serotype *enteritidis* was used in this study. The strain was isolated from food samples and was identified by 16S rRNA gene sequencing. It was routinely grown in Brain Hear Infusion (BHI) broth (Oxoid, Milan, Italy) at 37°C for 18-24 h. Overnight cultures of the salmonella strain were used to prepare serial dilutions containing from 10^8 cfu/mL to 1 cfu/mL. Ringer solution (Oxoid) was used as diluent. Plate counts were carried out by spreading 0.1 mL of the dilutions onto BHI agar (Oxoid) and by incubation at 37°C for 18-24 h.

Results and Discussion

A microcantilever resonator can be treated as a harmonic oscillator with dominant modes of vibration dependent just on the spring constant *k* and the effective mass *m*. Thus, when a substance is immobilized on cantilever surface, the consequent variation of resonant frequency Δf can be directly correlated to the added mass Δm as (Waggoner & Craighead, 2007):

$$\Delta m = -\frac{1}{2}\frac{\Delta f}{f_0} m \tag{1}$$

where f_0 is the resonant frequency before binding. Besides this simple relation was successfully applied for MC-based biosensing of small molecules like proteins (Oliviero et al., 2008; Ricciardi et al., 2010), some aspects must be carefully considered in case of cells adsorption: (i) the mass contribution from non-specific bindings (Waggoner & Craighead, 2007), (ii) the variable mass of the cells and, in particular, their water content (Davila et al., 2007), (iii) the stiffness and thickness of the added layer (Gupta et al., 2006), (iv) the position of cells on cantilever surface (Ramos et al., 2006). Therefore, we used Eq. 1 just as a rough indication of adsorbed mass, while the frequency shift was linked to sample concentration.

Fig. 1 reports a picture of the MC-based device for in liquid measurements (on the left) and the resonance spectra of the fifth mode of vibration (on the right) of a cantilever after antibody immobilization (Ab) and after flowing an aliquot of 1 mL of *Salmonella* suspension (10^5 cfu/mL). The clearly visible frequency shift of 660 Hz is the result of the changing in MC effective mass due to the immobilization of entire bacteria thanks to antibody binding with their membrane proteins. Using Eq. 1 we can evaluate that such effective mass is of the order of few tens on ng (10^{-8} grams); considering that a single *Salmonella* cell has a mass of approximately 1 pg (10^{-12} grams), we can assume that the number of cells immobilized on MC surface can be roughly estimated to be around 4000 units. We monitored first six flexural modes of vibration, but just the highest ones (fourth, fifth and sixth) exhibit a significant frequency shift. We are currently optimizing our device design, in order to develop cantilever with higher Q-factors (i.e. narrower curves). So far, our biosensors can easily detect the presence of pathogenic bacteria such as *S.* Enteritidis in concentration of 10^5 cfu/mL in just 40 minutes, without any enrichment and/or sample preparation. Such a concentration, although being still too high for commercial application, is comparable with previously reported literature data, where *Salmonella* cells have been successfully detected in liquid environment in the range 10^8-10^3 cfu/mL (Weeks et al., 2003; Zhu et al. 2007; Li et al., 2009). These measurements are presently made in cell suspension, but the methodology can be applied to the direct monitoring in raw materials and/or food matrix, rapidly and *in situ*. Nevertheless, we expect that working directly in food matrix respect to buffer suspension would cause a decrease of sensitivity of at least one order of magnitude, as reported by Maraldo et al. (2007) for the case of *Escherichia coli* detection.

If the goal of using cantilever-based biosensors in liquid is the real-time monitoring of food safety, performing the measurements in vacuum drastically increase the sensitivity of the technique. In the latter method, the MC array (a micrograph is reported in the inset of Fig. 1, on the left) is dipped in the solution for the bacteria incubation (typically for 1-2 hours), washed, dried and placed in a vacuum chamber. Thanks to the minimizing of the viscous effects of the environment, the cantilever vibrating in vacuum reveals the highest resonant frequencies and the greatest Q-factors, resulting in a significant enhancement of mass sensitivity. We characterized different cantilever arrays exposed to varying bacteria dilutions and we were able to successfully detect the presence of *S.* Enteritidis in concentration as low as 10^3 cfu/mL. Figure 2 reports an example of such measurements: on the left, the resonance spectra of the

first mode of vibration of a cantilever after Protein G binding (PtG), after antibody immobilization (Ab) and after incubation with an aliquot of 20 μL of Salmonella suspension (10^3 cfu/mL) is shown. On the right, another MC is used for a negative control experiment: the resonance curve is monitored after Protein G binding (PtG), after antibody immobilization (Ab) and after incubation with an aliquot of 20 μL of Ringer solution, without cells (blank). As can be clearly seen, while the former MC exhibits a remarkable negative shift due to cell immobilization, no considerable change in frequency is evident for the latter. This proof is crucial to state that non specific adsorption due to buffer solution has a negligible effect when compared to frequency shift due to *Salmonella* binding, even at concentration as low as 10^3 cfu/mL. Since we used MC with slightly different geometrical dimensions (due to inherent tolerances in the fabrication process) and more than one resonance mode is usually detected, we compare the experiments in terms of relative frequency deviation $\Delta f/f$ rather than absolute frequency shift. Than, we calculate the weighted average and uncertainty, as reported in (Ricciardi et al., 2010).

A preliminary control experiment (not included here as a figure) was performed where an array of four unfunctionalized MCs was inoculated into a *S.* Enteritidis suspension (10^3 cfu/mL): the average frequency deviation resulted of $\overline{\Delta f/f} = (0{,}1 \pm 2{,}7) \cdot 10^{-5}$. For the blank experiment, we dipped one Ab-immobilized MC in Ringer solution without bacteria: combining both first and second resonance modes we obtained an average deviation of $\overline{\Delta f/f} = (-4{,}1 \pm 0{,}3) \cdot 10^{-5}$. Finally, when incubating an array of seven Ab-immobilized MCs with 20 μL of 10^3 cfu/mL *S.* Enteritidis and monitoring both first and second resonance modes we obtained $\overline{\Delta f/f} = (-1{,}3 \pm 0{,}2) \cdot 10^{-4}$, successfully one order of magnitude larger than control experiments. If we now consider that approximately just 200 cells are suspended in the small volume used for such analysis, we can deduce that these shifts are caused by the adsorption of just few cells on each cantilever. In fact, dipping an array of 7 MCs (as the one depicted in Fig. 1) in a drop of 20 μL of 10^3 cfu/mL would statistically bring no more than 3 cells on each microstructure. Therefore, our biosensors have the potentiality of detecting the presence or absence of a single unit of a pathogenic bacterium, such as *S.* Enteritidis.

Conclusions

Two methods were reported for the detection of *S.* Enteritidis, a pathogenic bacterium, making use of antibody-immobilized microcantilever biosensors, a novel class of mass detectors. With the first one, we integrated the mechanical sensors on a microfluidic platform (lab-on-a-chip) to perform online analysis, directly in liquid environment. We showed that our portable biosensors could easily detect the presence of the pathogenic bacteria in concentration of 10^5 cfu/mL in just 40 minutes, without any enrichment and/or sample preparation. To increase the mass sensitivity of our analysis, we also fabricated microstructures optimized for vibrating in vacuum environment. Making use of a dip-and-dry technique we showed that in such configuration the experimental limit of detection is as low as 10^3 cfu/mL. Due to the extremely small volume needed, our biosensors show the potentiality of detecting the presence or absence of a single cell of a pathogenic bacterium, as demonstrated for *S.* Enteritidis.

Acknowledgements

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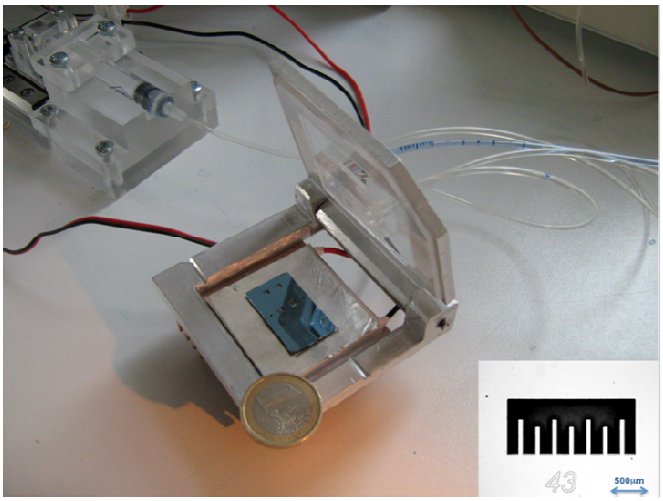
Legends

Figure 1. Left - A picture of the portable system (lab-on-a-chip) for measurements in liquid environment. In the inset, an optical micrograph of a typical cantilever array for measurements in vacuum environment. Right - In liquid MC resonant curve after antibody immobilization (Ab) and after 40 minutes flowing of *S.* Enteritidis suspension (10^5 cfu/mL).

Figure 2. Left - In vacuum MC resonant curve after protein G (PtG), antibody (Ab) and *S.* Enteritidis incubation (10^3 cfu/mL): the frequency shift is proportional to the added mass. Right - In vacuum MC resonant curve after protein G (PtG), antibody (Ab) and Ringer solution incubation (blank): for the latter, no appreciable frequency shift is detected.

Figure1



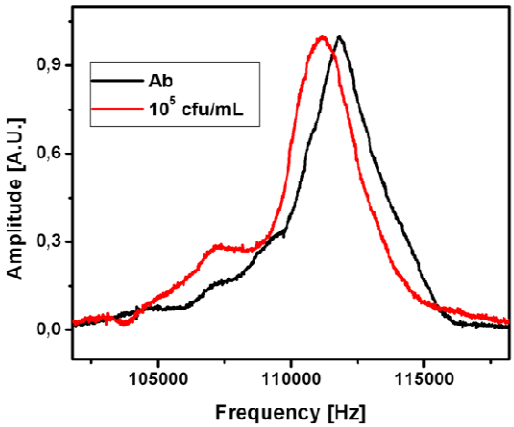
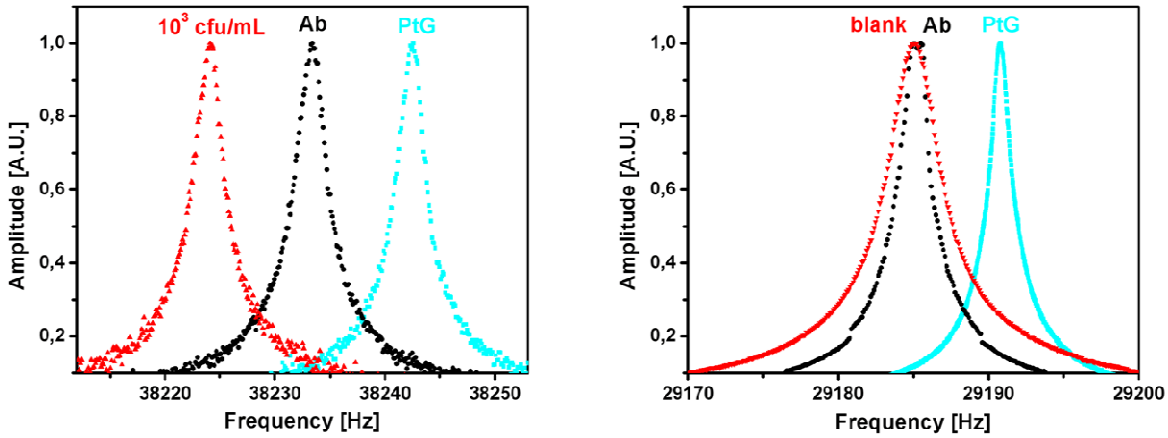

Ab
10^5 cfu/mL
Amplitude [A.U.]
0,0
0,3
0,6
0,9
105000
110000
115000
Frequency [Hz]

Figure 2


10^3 cfu/mL
Ab
PtG
Amplitude [A.U.]
1,0
0,8
0,6
0,4
0,2
38220
38230
38240
38250
Frequency [Hz]
blank
Ab
PtG
Amplitude [A.U.]
1,0
0,8
0,6
0,4
0,2
29170
29180
29190
29200
Frequency [Hz]